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MISCELLANEOUS

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Molecular Punctuated Equilibrium in the Evolution of *Rhopalura ophiocomae* (Mesozoa: Orthonectida) 18S rRNA, Hairpin 17

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Abstract—Alternative states of the predicted structure of hairpin 17 in the small-subunit ribosomal RNA genes are described. Each of these structures is characteristic of one or several phylons of animals. For example, one of the alternatives could be found in the rRNA of most Bilateria, but not in protists, fungi, plants, or diploblastic animals. A presumable secondary structure of the 18S rRNA hairpin 17 of a lower multicellular animal, *Rhopalura ophiocomae* (Mesozoa: Orthonectida), was constructed. It differs drastically from the one of other lower multicellular animals, i.e., Rhombozoa, Myxozoa, and Acoela. The evolution of rRNA fragments according to the principle of punctuated equilibrium is discussed.

Key words: 18S rRNA, hairpin 17, *Rhopalura ophiocomae* (Mesozoa: Orthonectida), evolution, molecular phylogeny, punctuated equilibrium

INTRODUCTION

Evolutionarily stable regions are present in the 18S rRNA of all living beings. Although the functional role of most of these sequences remains obscure, one may suppose that either they are involved in ribosome-mediated processes, or they play a significant role in supporting the specific structure of functionally important ribosomal domains. On the contrary, some other, evolutionarily variable rRNA regions, may differ even among closely related species, and the number of nucleotide residues therein is either strictly determined, or variable.

From this point of view, the region of the hairpin 17 of 18S rRNA [1] does not have any of these two types of structure, and demonstrates variable evolutionary stability.

EXPERIMENTAL

Fragments of the presumed secondary structure of the hairpin 17 of 18S rRNA of *Rhopalura ophiocomae* were constructed basing on the universal eukaryotic model [1] and two data sets obtained independently (GenBank X97157 and U58369) [2, 3]. All other sequences were also taken from GenBank, and their numbers are given in figure captions.

RESULTS AND DISCUSSION

Hairpin 17 Structure

Hairpin 17 is present in all the small-subunit rRNAs of pro- and eukaryotes [1]. It includes residues corresponding to nucleotides 628–659 of the *Homo sapiens* 18S rRNA (GenBank M10098).

The structures of hairpin 17 in the rRNAs under study largely fall into two groups, with some exceptions. The first group includes those of protists, algae, higher plants, fungi, and diploblastic animals (sponges, jelly-fishes, coelenterates) (Fig. 1A). The stem of its distal part is 10 bp long (the sum of regions I, II, and III in Fig. 1). The second one could be observed in Bilateria, including such distantly related groups as flat worms, nemertinas, chordates, echinoderms, tentaculates, chaetognates, priapulids, kinorhynchs, annelids, mollusks, and other (some of them are presented in Fig. 1B). In the second group of structures the distal part of the stem is asymmetrical ("10.5 bp" long) owing to the presence of an additional free guanine (or, very rarely, adenine) nucleotide located in a definite hairpin site, designated *17a* (Fig. 1B). Irregularly, an A-C pair is present adjacent to *17a*.

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As the first type of structure (A) is widely distributed among eukaryotes including diploblastic animals, and the second type (B) may be found only in Bilateria, the first type may be regarded as an ancestral one, and the second one, using the terminology of cladism, as an apomorphy [4]. Evolutionary transition from the first type to the second one was not accompanied by pronounced changes in the primary structure of the latter, thus allowing identification of homologous regions (dotted lines in Fig. 1).

The evolutionary A–B transition draws attention to the study of phylons which are presumably close to the root of Bilateria. One may imagine that some of them have already acquired the B type, whereas some other, evolutionarily older groups still have the ancestral A type. Formerly, one of such groups, the Orthonectida, has been considered as one of the classes of Mesozoa, a transitional group between single-cell and multicellular organisms [5, 6]. Recent comparative studies of 18S rRNA structure demonstrated that Orthonectida are close to Bilateria [2, 3], but the exact phylogenetic links remain obscure.

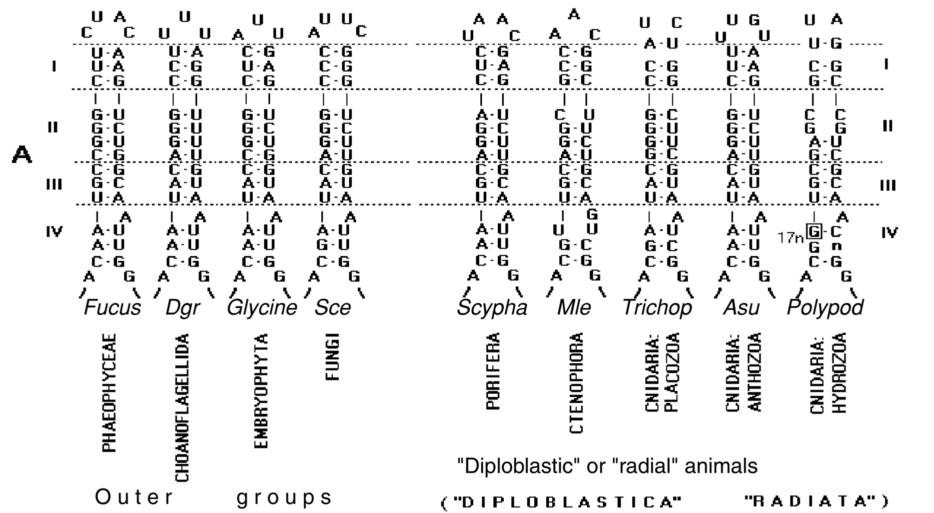
The nucleotide sequence of hairpin 17 of Orthonectida substantially differs from that of the A and B structures; its secondary structure is still undetermined. We suppose that to predict its structure is a complex problem. It cannot be solved by direct calculations of the difference in the free energy of unpaired and locally double-stranded structures, because some regions of the ribosomal RNA interact with proteins and with other parts of the RNA molecule distant in the primary structure but spatially close in ribosomes.

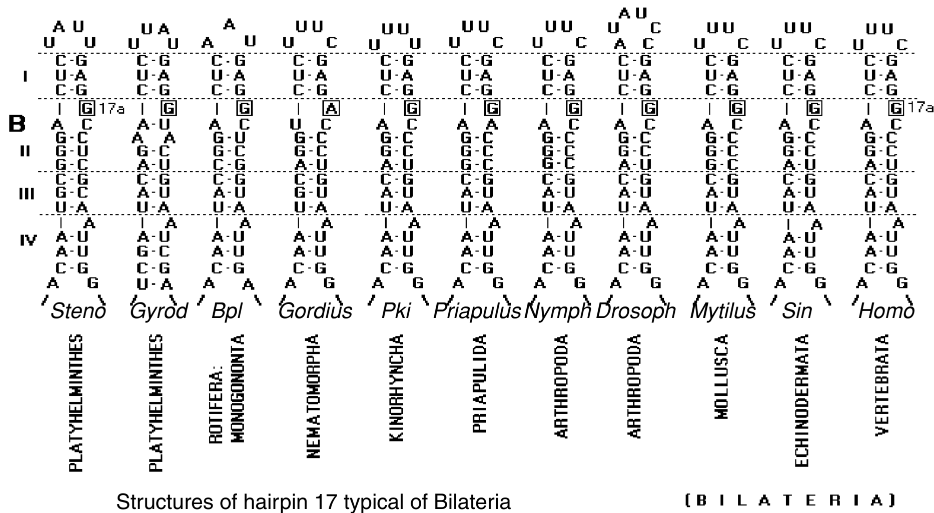
As a result of such interactions, a region *in vivo* may have a secondary structure different from the optimal one, calculated on the basis of local sequence. In our opinion, physicochemical characteristics of double helices should be supplemented with comparative data on the evolutionary stability of the structure elements under study. If a predicted double-helical region does exist *in vivo*, then the interspecies differences in one RNA strand must be compensated for in the complementary strand [7].

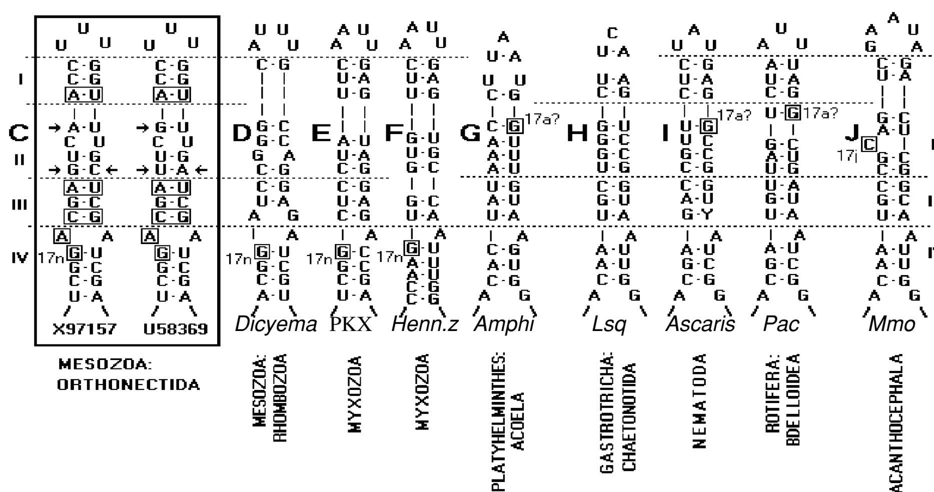
Direct comparison of Orthonectida hairpin 17 with the corresponding structures of other animals is hindered by the gross differences in primary structures and the possibility of at least two alternative but energetically suboptimal modes of compactization of this region in Orthonectida. Quite unexpectedly, comparison of two independently determined Orthonectida sequences [2, 3], both formally coming from one and the same species, *Rhopalura ophiocommae*, proved to be very informative. They differ by less than 5%, but three substitutions were detected in hairpin 17: A ⟷ G transition (corresponding to residue 513 in X97157), and two transversions, G ⟷ T (position 510) and C ⟷ A (position 526) (Fig. 2). *In toto*, 21 transversions were found in the gene, i.e., one per 89 nucleotides, but their relative concentration in the region under study is much higher (2 transversions per 17 nucleotides). The accidental independent origin of these substitutions seems to be hardly probable. The probability increases if the residues are from the double-stranded region, because the substitution could be compensatory [7]. If so, transversions in positions 510 and 526 could not be considered independent; on the contrary, these two events could be due to a single transversion in this region. It should be mentioned that in both alternative states the two residues may form a Watson–Crick pair. We supposed that the substitutions do not alter the secondary structure of hairpin 17, which seems to be identical in both representatives of one species *Rh. ophiocomae*. If residues 510 and 526 do really form a pair in the stem of the hairpin, we can easily obtain the structures presented in Fig. 1C. These structures appeared to be energetically optimal (–14.1 kcal/mol for X97157 and –12.0 kcal/mol for U58369). In the case of X97157, the transition of residue 513 does not contradict this model. The substituted residue is in the canonical AU pair, and in the case of U58369 it is in the permissible GU one (Fig. 1C).

Summing up, there exists some indirect evidence in favor of the structure of hairpin 17 of *Rh. ophiocomae* presented in Fig. 1C. However, this is insufficient to declare that such a structure exists *in vivo*. Let us consider the arguments against the alternative models of the secondary structure of *Rh. ophiocomae* hairpin 17. We shall begin with the analysis of the structure of the basal part of the hairpin and the adjacent regions in the ordinary structure, and its presumable modification in *Rh. ophiocomae*.

The basal part of the double-stranded stem of hairpin 17 (region IV in Fig. 1) of animals and of many other living beings is, as a rule, composed of three nucleotide pairs. Anteriorly, an unpaired consensus sequence 5'-AAAUAA-3' is adjoined to it (Fig. 3). On the opposite side, it is flanked by a single-stranded spacer sequence between hairpins 17 and 18. As a rule, this spacer is six nucleotides long (5'-GAAUGA-3') (Fig. 3). These elements are evolutionarily rather conservative, and the rare deviating structures are easily deduced from the parental one. For example, all three elements of this region of the *Gyrodactylus salaris* rRNA [8] seem to be different, because the left and the right spacers are shorter by one nucleotide and the stem has an additional nucleotide pair (Fig. 3). However, this structure may be easily obtained from the consensus if one assumes that mutations of two nucleotides at the borders of the region produce the complementary pair. Nevertheless, the formal possibility of the existence of such a pair on a plane does not imply that it is present in ribosomes, where the spacer may interact with a ligand (rRNA or protein). Moreover, it may depend on the length of the spacer,







Atypical structures of hairpin 17 of Bilateria lacking indisputable *17a* apomorphy

because hairpins 17 and 18 should be specifically spatially oriented.

The anterior 5'-AAAUG-3' sequence flanking the hairpin 17 of *Rh. ophiocomae* rRNA is similar to the consensus. As in other organisms, these residues may be intimately connected with the stem, and the last adenine residue of the left spacer is substituted by an uracil residue, which is a real or virtual member of a base pair, as in the hairpin 17 of *G. salaris*. The residues directly following the uracil one correspond to the consensus 5'-CRR-3' of the anterior branch of the basis of the stem (Fig. 1), supporting the idea that the stem originates from these nucleotides. On the contrary, an alternative hypothesis, according to which the residues following the 5'-AAAUG-3' sequence are not involved in the formation of the hairpin 17 stem, produces an exceptionally long spacer. For example, if the 5'-CRR-3' motif is employed, starting from position 507 in X97157, this will extend spacer by four nucleotides. Such a version should be rejected until additional arguments supporting it are found.

The arguments discussed above are of importance for proper positioning of the anterior branch, because many other evolutionarily conservative residues in the *Rh. ophiocomae* sequence, which are important in the search for homology of hairpin 17 elements, are changed. For example, 5'-ACC-3' substitutes for the consensus 5'-CUC-3' in region I, and 5'-CGA-3' replaces 5'-URC-3' in region III (Fig. 1C). An "additional" A-506 at the border of regions III and IV attracts attention: it is unique for Orthonectida; analogous "additional" nucleotides can be found only in the nematode *Pelliodítis typica* [9].

Let us now analyze the posterior branch of the *Rh. ophiocomae* hairpin 17. Its sequence differs substantially from the sequences of this region in the majority of organisms. It lacks the characteristic 5'-GYA-3' motif (Fig. 1, region III), but a short direct repeat of four nucleotide residues, 5'-UCGA-3', or an overlapping repeat of six nucleotides, 5'-GAUCGA-3', appear in it (U58369) (Fig. 2). Other species do not have direct repeats in this region. It is evident that the shift of the posterior branch by four nucleotides relative to the anterior one will result in the same complementary pairing in the region of the repeating unit and, if one does not take into account the neighboring nucleotides, the choice between the two alternatives is impossible. However, if we shift the anterior branch by four nucleotides relative to the model structure in Fig. 1, then the fifth position from the base of the hairpin (or the fourth, if we do not take into account the basal pair) in the X97157 sequence will be occupied by a cytosine residue. However, this site at the border of region III in other species is occupied by unpaired A, or, occasionally, by G. This is the most conservative element of hairpin 17. We failed to observe a single Pu–Py change at this site among two hundred analyzed eukaryotic sequences of different origin. In addition, if the shift occurs, the right spacer between hairpins 17 and 18 becomes four nucleotides longer, which has also never been observed. Hence, such an option of the secondary structure should be rejected.

The presence of an unpaired purine residue at the border of the two regions of the hairpin should be taken into account when the posterior branch is shifted not by four, but by any other number of nucleotide residues relative to the anterior branch. The *Rh. ophiocomae* rRNA is enriched in pyrimidine nucleotides in the vicinity of A-530 (as in X97157). The only other candidate may be the adjacent G-529. However, the shift of the posterior branch by one nucleotide causes multiple disorders in the complementary structure of the hairpin, and thus should be discarded.

Some models that should be rejected are shown in Fig. 4. The hairpin 17 in this figure is represented by one and the same option, in which the posterior branch is shifted by four nucleotides. Although the stem in this case forms a nearly perfect double helix, it does not suffice because of the absense of a conservative unpaired purine residue at the border of regions III and IV. The difference between the models depends on to which secondary structure elements the nucleotides "liberated" as a result of the shift belong. If they occupy the place in the spacer between hair-

←

Fig. 1. A hypothetical secondary structure of the 18S rRNA hairpin 17. Homologous regions of the hairpins are separated by broken lines and marked by Roman numbers. Nucleotide residues characteristic to a specific group are in frames. Short arrows indicate sites differing in two independently determined *Rh. ophiocomae sequences.* The following sequences with the GenBank numbers were analyzed: A: Phaeophycea, *Fucus gardneri*, X53987; Choanoflagellata, *Diaphanoeca grandis*, L10824 (*Dgr*); Embryophyta – *Glycine max*, X02623; Fungi – *Saccharomyces cerevisiae*, J01353 (*Sce*); Porifera, *Scypha ciliata*, L10827; Ctenophora, *Mnemiopsis leidyi*, L10826 (*Mle*); Placozoa, *Trichoplax* sp., Z22783 (*Trichop*); Cnidaria, *Anemonia sulcata*, X53498 (*Asu*); Cnidaria, *Polypodium hydriforme*, U37526 (*Polypod*); B: Platyhelminthes, *Stenostomum* sp., U95947 (*Steno*); Platyhelminthes, *Gyrodactylus salaris*, Z26942 (*Gyrod*); Rotifera (Monogononta), *Brachionus plicatilis*, U29235 (*Bpl*); Nematomorpha, *Gordius aquaticus*, X87985; Kinorhyncha, *Pycnophyes kielensis*, U67997 (*Pki*); Priapulida, *Priapulus caudatus*, X87984; Arthropoda, *Nymphon* sp., U88338 (*Nymph*); Arthropoda, *Drosophyla melanogaster*, M29800 (*Drosoph*); Mollusca, *Mytilus edulis*, L24489; Echinodermata, *Strongylocentrotus intermedius*, D14365 (*Sin*); Vertebrata, *Homo sapiens*, M10098; C: Mesozoa: Orthonectida, *Rhopalura ophiocomae*, X97158 и U58369; D – J: Mesozoa: Rhombozoa, *Dicyema orientale*, D26529; Myxozoa, PKX, U79623; Myxozoa, *Hennegua zschokkei*, U13827 (*Henn.z*); Platyhelminthes: Acoela, *Amphiscolops* sp., D85099 (*Amphi*); Gastrotricha, *Lepidodermella squammata*, U29198 (*Lsq*); Nematoda, *Ascaris* sp., M38348; Rotifera (Bdelloidea), *Philodina acuticornis*, U41281 (*Pac*); Acanthocephala, *Moniliformis moliniformis*, Z19562 (*Mmo*).

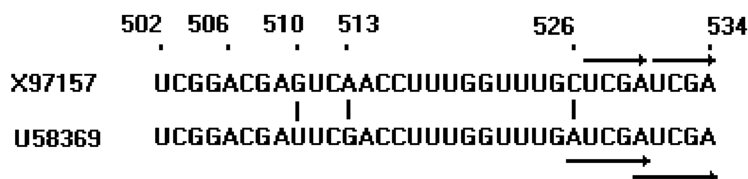


Fig. 2. Primary structure of the *Rh. ophiocomae* 18S rRNA hairpin 17.

pins 17 and 18 (Fig. 4, left), then the spacer becomes too long. This fact, together with the absense of the conservative purine residue in the hairpin 17, rules out such a model. If one supposes that the distal nucleotides of the spacer are in the hairpin 18 (Fig. 4, right), then the spacer becomes too short and its nucleotide sequence will not be like the consensus, i.e., 5'-GAAUGA-3', and the conservative sequence 5'-AACGA-3' will not occupy its fixed position relative to hairpin 18 (Fig. 3, indicated by arrow).

This reasoning allows us to suggest the structure of hairpin 17 and of the spacer separating it from hairpin 18 in the small-subunit rRNA of *Rh. ophiocomae*. The hairpin 18 always includes 21 residues even if its primary and, perhaps, secondary structure is modified, as in the PKX myxosporidium rRNA [10] (Fig. 3). Commonly, the 17-nt loop of hairpin 18 is “locked” by two nucleotide pairs [1]. In rare cases, for example, in *Rh. ophiocomae*, in diciemids, and in some other invertebrates, one of these pairs “unfastens” (Fig. 3) and the hairpin includes 19 rather than 17 unpaired nucleotides (Fig. 3). Hence, the modified hairpin 18 of *Rh. ophiocomae* is easily derived from the standard one in such a way that the “additional” nucleotide in the spacer between hairpins 17 and 18 cannot originate from hairpin 18. Hence, the 7-nt spacer of *Rh. ophiocomae* (5'-AGAUGGA-3') is a derivative of the standard 6-nt spacer 5'-GAAUGA-3', and is formed by insertion of an “additional” nucleotide inside this sequence rather than by addition of residues from the flanking sequences. The insertion must have occurred in position 538–539, to the right of the conservative residue U-537 (X97157).

The first of the nucleotides of the spacer (position 534) may form a complementary pair with residue 502 and be a component of the double helix located at the basis of the stem 17. As in ordinary structures, the six nucleotides of the spacer remain free. Hence, if the spacer loses one nucleotide residue inserted in hairpin 17, this is compensated by insertion of one additional nucleotide. This is a circumstantial evidence supporting the idea that elongation of the basal part of hairpin 17 by one nucleotide pair is a real rather not a virtual event, as it is in *G. salaris*.

Variability of Hairpin 17 Structure in Animals

In the majority of Bilateria the structure of the 18S rRNA hairpin 17 is very similar. Among the indubitable Bilateria, some deviations from the classical B type, in addition to those presented in Fig. 1G–J, are observed in tardigrades [11–14], pogonophores [15], and some crustaceans [16, 17]. In these animals the distal part of hairpin 17 is longer than in the type B structure, and it is rather easy to indicate the purine residue homologous to *17a* (except the one in tardigrades). The evolution of this region in lower Bilateria is more difficult to understand. If the structure of hairpin 17 deviates from the typical one in these species, its distal part is always shorter than in higher Bilateria and resembles the one in their ancestors, the Radiata (Fig. 1C–J). The unpaired purine residue in such cases is lost. This structure may be explained by conservation of the ancestral state, or by secondary reversion to it.

In morphologically “primitive” Bilateria (flat worms, except acoelic turbellarias and gnathostomulides) hairpin 17 has an ordinary structure and includes the “additional” unpaired purine residue. Hence, the phylogenetic lines that are “younger” than flatworms and do not possess this character must have lost it in evolution. Such a process has undoubtedly had occurred in rotifers and gastotrichs, where, within one type, species have either the typical or the modified hairpin 17. A sort of “morphological succession” may be suggested, starting with the rotifer *Brachionus plicatilis* [14, 18], with a hairpin 17 of the ordinary structure (Fig. 1B), proceeding to *Philodina acuticornis* (Bdelloidea) [19] which lost the asymmetry of the distal part of the hairpin (Fig. 1I), and then coming to thorny-headed worms (Fig. 1J) with the newly formed asymmetrical distal part. Such evolutionary transformations are in accord with the hypothesis of the monophyly of rotifers and thorny-headed worms, based on the comparative anatomy data [20–22] and finding support in the results of the complete sequencing of the 18S rRNA [18], according to which thorny-headed worms originate from Bdelloidea rotifers [19].

As in other Bilateria, there is a guanine residue at the border of regions I and II of the *Ph. acuticornis* hairpin posterior branch, and an uracil counterpart in the anterior branch. However, in the outer groups (protists, diploblastic animals) this site may be occu-

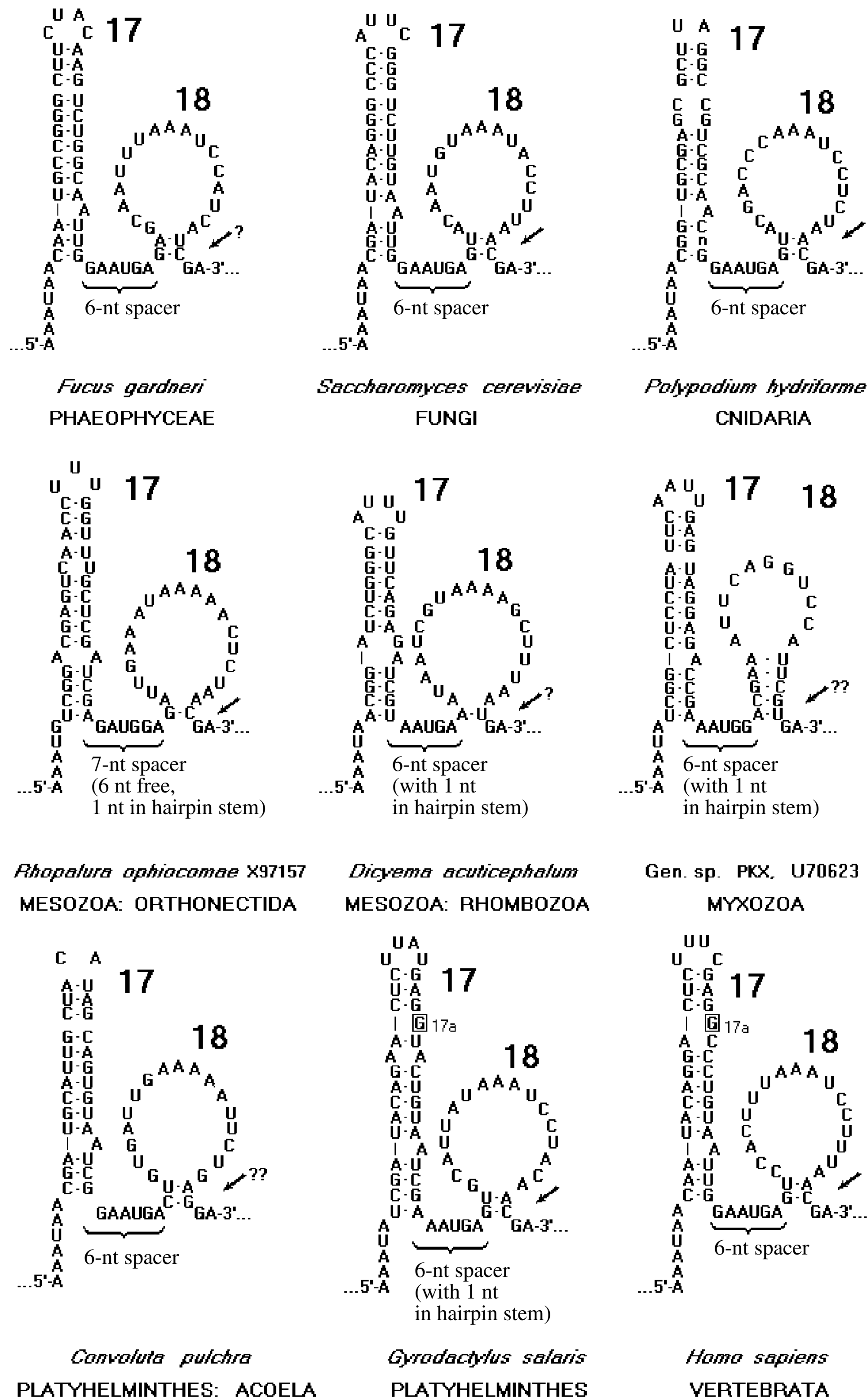


Fig. 3. Presumable secondary structure of the flanking regions of the 18S rRNA hairpin 17. Long arrows indicate a conservative sequence 5'-AACGA-3'; question marks indicate deviations of homologous sequence of a species from the consensus. Mesozoa: Rhombozoa, *Dicyema acuticephalum*, D26530; Platyhelminthes: Acoela, *Convoluta pulchra*, U70086. GenBank numbers of other species are given in the legend to Fig. 1.

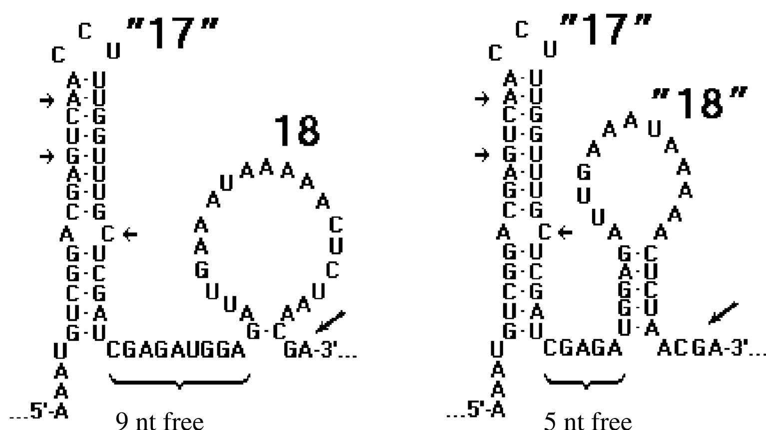


Fig. 4. Examples of nearly perfect helices in the region of the 18S rRNA hairpin 17 of *Rh. ophiocomae* rejected basing on comparative morphology reasons. Short arrows indicate the sites differing in X97157 and U58369 sequences. Long arrows mark the conservative sequence 5'-AACGA-3'.

pied by any nucleotide except G (Fig. 1A). Basing on these observations, one may suggest that the paired G of *Ph. acuticornis* (*17a?*) originates from the unpaired *17a* by deletion of one nucleotide following it in the posterior branch.

The characters of the *Ph. acuticornis* hairpin 17 may be observed also in a large group of Bilateria, the nematodes. The distal part of hairpin 17 in this group is 10 bp long (the sum of regions I, II, and III), as in diploblastic animals and *Ph. acuticornis*, but, as opposed to diploblastic animals, the G residue occupies the site at the border of regions I and II. Such similarities suggest that the paired G residue *17a?* of nematodes originated in the same way as in *Ph. acuticornis*.

The secondary structure of Acoela rRNA hairpin 17 is like that of nematodes and the rotifer, *Ph. acuticornis*, although with some reservations. Two Acoela species, *Convoluta naikaiensis* and *Amphiscolops* sp., (Fig. 1G) possess the G residue resembling *17a*, though in *Convoluta pulchra* this site is occupied by C (Fig. 3). In essence, the *C. pulchra* hairpin 17 is like the one of plants, fungi, and diploblastic animals, although it has a modified sequence in region I of the stem and a shortened loop. Last, the Acoela hairpin may be taken as a unique structure, in which only 3 bp correspond to the stem region II, whereas the apical part is of canonical dimensions. Such a structure may be derived either from A or from B: in both cases two deletions in the opposite strands of the hairpin are necessary. The proper choice of one of the three ways of homologization of the Acoela hairpin 17 elements cannot be made as yet. The Acoela remain the only group of commonly accepted Bilateria with no direct evidence whether the similarity of their hairpin 17 with that of their ancestors, the diploblastic animals, is a secondary one. The structure of hairpin 17 of Acoela may be taken as an argument confirming that this clade is one of the earliest among Bilateria [23]. The same is true for the gnathostomulid hairpin 17 [24], because there is no unpaired purine residue.

In addition to Acoela and Gnathostomulida, the *17a* character is not found in three other groups: Orthonectida, Dicyemida and Myxozoa, presumably belonging to Bilateria, and no traces of its presence in the past could be observed. The simplest explanation is that these groups branched off before the *17a* character appeared in the evolution of Bilateria; the alternative explanation is that they have lost it again.

The data available do not allow one to choose the correct option. On the one hand, the morphology of the species of the above-mentioned groups substantially differs from that of Bilateria. On the other hand, Acoela, Orthonectida, Dicyemida, and Myxozoa have accumulated many substitutions in their 18S rRNA genes, being in this aspect among the leaders in Metazoa. It seems obvious that the region of hairpin 17 could be among the regions with secondary modifications. The unique properties of this region in all these groups, differentiating them from both Bilateria and Radiata, support this hypothesis. Moreover, substantial differences have been registered even within Myxozoa (Fig. 1E,F), suggesting that the hairpin 17 evolution still proceeds in this group. In such a situation it will be premature to exclude the possibility of the second loss of the *17a* residue. It is no mere chance that the second loss of the *17a* is observed in the lines with the rapidly evolving 18S rRNA gene, such as nematodes and the Bdelloidea rotifers [9, 19]. For example, in *Lepidodermella squammata* the purine residue *17a* not only could disappear, but this site at the border of

regions I and II might be occupied by a pyrimidine residue, as it is in coelenterates (Fig. 1H).

What is the phylogenetic relatedness of the organisms with unique hairpins 17? Do they form a monophyletic group? When traditional phylogenetic tree construction programs are used on complete 18S rRNA sequences, these groups sometimes get together, with a high bootstrap index (not shown). However, such a result is not stable and depends strongly on the set of the species analyzed. It is well known that the sequences of 18S rRNA of Acoela, Gnathostomulida, Orthonectida, Dicyemida, and Myxozoa deviate substantially from ordinary sequences. Notably, the existing programs tend to erroneously unite "too long" branches [25, 26], which calls into question the very fact of unification of these taxa. In the region of hairpins 17 and 18 of this group, we did not observe a single indubitable synapomorphy, although it has a probable symplesiomorphy: the absense of the *17a* purine residue. Another group, Orthonectida + Dicymeida (=Mesozoa), has a synapomorphy in this region: the loss of complementarity in the apical part of the stem of hairpin 18. However, it seems premature to conclude that this is a unique character. For example, in the *Paramecium tetraurelia* 18S rRNA hairpin 18, a similar transition of one nucleotide pair from the stem to the loop occurs (not shown). In the Mesozoa + Myxozoa group, the basal part of the hairpin 17 is elongated, but by some other mechanism. In Orthonectida, the basal part of the hairpin is elongated by insertion of one nucleotide residue from the spacer between hairpins 17 and 18, which is compensated by parallel elongation of the spacer, but in Dicymeida and some Myxozoa the insertion of the nucleotide from the spacer is not accompanied by compensation (maybe it is not realized *in vivo*?). In other Myxozoa (Fig. 1F) an additional nucleotide pair appears *de novo* within the hairpin 17 stem. Finally, the group Mesozoa + Myxozoa and a parasitic coelenterate *Polypodium hydriforme* have a rare transition A ⟶ G in the position 505 of *Rh. ophiocomae* (X97157) (Fig. 1, *17n*). Formerly, basing on molecular data, *P. hydriforme* was considered as a sister group for all Bilateria [27]. However, all Bilateria have a plesiomorphy in this site. Hence, *17n* could have originated independently in these species.

The structures of the *Rh. ophiocomae* hairpins 17 and 18 cannot be easily treated in terms of phylogenetics. The structure of hairpin 17 in all the groups transitional from Radiata to Bilateria cannot be attributed with confidence either to type A or to B (Fig. 1). This is observed in Orthonectida (two sequences), Dicyemida (three sequences) [2, 28], Myxozoa (20 sequences) [27, 29–33], Acoela (three sequences) [34, 35], Nemertodermatida (two species) [35, 36], and Gnathostomulida (one species) [24].

The variability of this region in the transitional groups contrasts its stability among classical Bilateria (Fig. 1B). It can hardly be accidental that the hairpin 17 in transitional groups does not have a clear-cut "bilaterial" structure. On the other hand, if the hairpin structure is compared in various types of animals, we cannot but assume that the *17a* character in lower Bilateria has more freedom to revert to the ancestral state.

"Punctuated Equilibrium" in RNA Evolution

Summing up, the 18S rRNA hairpin 17 region cannot be considered as an evolutionarily conservative or variable one; it belongs to the third group with "intermediate" conservatism, but this definition is not exact. In fact, the evolutionary conservatism of the hairpin 17 is not constant and changes in evolution. This region may persist constant for a long time, with no substantial changes in its structure, but sometimes temporal instability of this region arises. The new modified state is fixed, and is then inherited in the succession of species. Hairpin 17 is a classical region of variable evolutionary conservatism. This conclusion follows from the fact that in a large set of species only a few types of organization of this region are detected. Following [37], we call this phenomenon "molecular punctuated equilibrium."

Some other regions of the 18S rRNA molecules are known which resemble hairpin 17. Being identical in many living beings, they may spontaneously change in an evolutionary line and then remain conserved in the new modified state. Eukaryotic and prokaryotic rRNAs may be a good example of such alternative states. Some specific, modified 18S rRNA structures have been reported to exist among invertebrates characteristic of monophyletic clades of different rank: of all higher multicellular animals (Tetraradiata), of all Bilateria, of some taxa of insects, of roundworms, etc. Specific conservative substitutions, small deletions or insertions in these groups are located not only in hairpin 17, but also in the helical regions 42 and 44 [38] and hairpin 49 [39]. More extended insertions have been also reported [40–43].

The punctuated evolutionary conservatism is somewhat an unexpected phenomenon, if one assumes that rRNA is composed of functionally more or less significant regions. One can hardly imagine that the *17a* unpaired purine residues are responsible for an indispensable function in Bilateria, but do not function in coelenterates and were repeatedly lost by the rotifer, *Ph. acuticornis*. On the other hand, not all but the majority of "punctuated equilibrium" cases are nothing but the appearance and disappearance of nucleotides or nucleotide pairs in a double helix or in a loop of fixed size. This is the case with the *17a* character in hairpin 17, with the loss by some nematodes

of the *17e* nucleotide pair in the same hairpin [9], with the origin of unique characters in the *Rh. ophiocomae* hairpin 17, with noncompensated deletions in the posterior branch of the hairpin 42 [38], and other analogous changes. Such mutations cause changes in the rRNA secondary structure and, to exclude structural alterations of ribosomes, they must be compensated by changes in a ligand interacting with this region, i.e., a protein or other rRNA region. This may take place on the principle operating in compensatory mutations in rRNA double-helical regions [7], but compensation must occur at a higher structural level. The frequency of neutral mutations altering the local rRNA structure depends on the probability of the two corresponding mutations in different loci, and appears to be very low, allowing them to be used as markers of cladogenesis.

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Note added in proof: Proceeding with our work, we determined the nucleotide sequence of the 18S rRNA gene of *Intoshia* (Orthonectida). It appeared that the structure of hairpin 17 of this species is in accord with the suggested model, although 10 nucleotide substitutions were registered in the region under study.

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